THE STREPTOCOCCI OF THE BOVINE UDDER

IV. STUDIES OF THE STREPTOCOCCI *

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The streptococci offer an interesting and important field for study because among them are found harmless and even useful organisms as well as extremely virulent ones.

Milk is perhaps the most important harborer of the harmless streptococci, but it may at times contain pathogenic forms. While milk cannot be considered a source of any micro-organism, it is an ideal food for most forms and may therefore contain many kinds of streptococci with which it has been contaminated.

In studying the literature on the streptococci we have been impressed with the extensive researches which have been conducted in this field, but felt that it was difficult from the results to obtain a clear conception of the common types of streptococci in milk. In the older studies tests were used for purposes of classification which varied from those used in later work. Often the classification was based largely on morphology and blood-plate reactions, and in other cases largely on the fermentation of test substances. Furthermore, selective methods of isolation have added other complications.

The confusion as to the streptococci in milk has led to various interpretations as to their significance in it and in other dairy products. Their presence has been considered by some authorities as an indication of fecal contamination from the cow and by others as an indication of inflammation of the udder. In such cases the presence of the streptococci was considered the significant thing, and no attention was given as to what kind they might be. It has been felt that further studies of the streptococci, using all the most valuable physiologic tests for their differentiation, would prove valuable. For this reason the studies reported in this paper were undertaken.

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* The preceding articles of this series were:

I. The Thermal Death Point and Limiting Hydrogen-Ion Concentration of Pathogenic Streptococci, Jour. Infect. Dis., 1918, 23, p. 290.

II. The Production of Ammonia and Carbon Dioxide by Streptococci, ibid., 1921, 29, p. 235.

III. Differentiation of Hemolytic Streptococci from Human and Bovine Sources by the Hydrolysis of Sodium Hippurate, ibid., 1922, 30, p. 388.

In this paper we shall take up the streptococci of the udder and in later papers the streptococci from other sources as well as those found in milk at various stages of souring. No attempt has been made to review the literature completely, for this has been done quite thoroughly by other investigators.

METHOD OF ISOLATING CULTURES

Samples of milk were obtained by milking directly into sterile tubes. In every case the first two or three streams were rejected. Composite samples were taken which included each quarter of the udder.

The samples were then taken to the laboratory and plated on blood agar. The agar had the following composition: 500 cc infusion broth, 10 gm. peptone (Fairchild), 5 gm. NaCl, 500 cc distilled water, and 15 gm. shred agar. The reaction was adjusted to P_H 7.5.

This medium was sterilized in tubes in 12 cc amounts. At the time of plating the agar was melted and cooled to 45 C., and from 0.66 to 1 cc of defibrinated horse blood was added to each tube of agar before it was poured into the plates. This method is substantially the same as that described by Brown [1].

After 48 hours' incubation at 37 C., cultures were made from plates representing each sample of milk from a cow. About 10 colonies which appeared to be streptococci were fished and inoculated into infusion-broth tubes and the cultures examined microscopically after 24 hours' incubation at 37 C.

Cultures were considered streptococci when chains of four or more cells were present in sufficient numbers to show that this grouping was not accidental. After microscopic examination the cultures were replated and then transferred to agar slopes for stock.

CULTURAL METHODS

Hemolysis.—This was determined by plating on the blood agar previously described, and the results were recorded according to the methods of Brown. The plates were incubated at 37 C. and examined after 24 and 48 hours and again after 24 hours' refrigeration.

The beta type of reaction on blood plates as described by Smith and Brown [2] consists of a sharply defined clear zone about the colonies. Under the microscope no blood corpuscles are seen near the colony.

[1] Monograph 9, Rockefeller Institute for Medical Research.
[2] Jour. Med. Research, 1915, 31, p. 455.

The alpha type of Smith and Brown is quite different. After 48 hours' incubation at 37 C., the zone about the colonies of 1 - 2 mm. appears greenish, with a partial hemolysis or decolorization of the blood corpuscles near the colony. After refrigeration for 24 hours a clearer zone appears beyond the partly hemolyzed or decolorized zone. The corpuscles in the outer zone are decreased in number. The size of the second clearer zone varies with different cultures, and it may be seen sometimes with the naked eye. The colonies should be examined, however, with a low power lens.

The gamma type of streptococcus described by Brown is one whose colonies do not produce any perceptible hemolysis or decolorization of the blood medium during incubation or refrigeration. Among our cultures of streptococci were many which produced no change other than a greenish color in the medium about the colony, and these are designated as gamma-G types. Brown found that all of his green-producing strains could be included in the alpha type, but many of our green producers did not show sufficient indication of alpha hemolysis to permit them to be included with this type.

Methylene Blue Test.—In this test whole milk was sterilized in tubes in 10 cc amounts. A 0.1% solution of medicinal methylene blue was also prepared and sterilized, then 1 cc of this solution was added to each tube of sterile whole milk. The tubes were shaken and incubated before inoculation to insure sterility. After inoculation the reduction of the dye and the condition of the milk were recorded each 24 hours for a period of 7 days.

Fermentation Tests.—The basic medium had the following composition: 10 gm. peptone (Bacto), 10 gm. yeast (Cerevisine, French preparation), and 1000 cc distilled water. The reaction was adjusted to about P_H 7.5.

To this basic medium 1% of the various test substances was added, and the medium was then sterilized. The hydrogen-ion concentration was determined by means of indicators after 7 days' incubation at 37 C. with dextrose, lactose, saccharose, and salicin, but after 14 days with mannite, raffinose, and inulin.

Carbon Dioxide and Ammonia Tests[3]—The ability of the streptococci to produce carbon dioxide and ammonia was determined by the method described by Ayers, Rupp, and Mudge[4]. For the production

[3] We are indebted to Dr. Philip Rupp of these laboratories for making the ammonia determinations and the tests for the hydrolysis of sodium hippurate.

[4] Jour. Infect. Dis., 1921, 29, p. 235.

of CO_2 from peptone medium A was used, and medium S for CO_2 from dextrose, both in 15 cc amounts. Eldredge tubes were used for the measurement of the amount produced. The production of ammonia was determined in medium B by the Folin method. The composition of these mediums is given in the paper of the authors just quoted.

Sodium Hippurate Test.—In the determination of the hydrolysis of sodium hippurate, the cultures were grown in the peptone-hippurate medium described by Ayers and Rupp.[5] When the organisms grew with difficulty, beef infusion broth with sodium hippurate was used. The hydrolysis of the hippurate with the formation of benzoic acid was determined in most cases by distillation, as described by Ayers and Rupp, but with some of the cultures the ferric chloride and acid test was used as described by the same authors.

CULTURAL CHARACTERISTICS OF THE STREPTOCOCCI OF THE UDDER

Most of the cultures in our collection came from the udders of apparently normal cows. A few, however, were from cows having mastitis. These cultures were not separated from the others, because we have not found any particular organism to be characteristic of inflamed udders. The types found in the normal udders were the same as in the cows with mastitis, the only difference being in the larger numbers found in cases in which mastitis was present. The 100 cultures studied were obtained from 55 samples of milk taken from 54 cows in two herds near Washington. One cow was examined twice.

Instead of recording in this paper the cultural characteristics of every culture, similar cultures have been grouped together, as is shown in table 1. It must be realized that not every culture showed the exact P_H values here given for fermentation test. There was of course a slight variation, but the figures represent the general limits reached. The range in cc of CO_2 from 15 cc of medium is given. For NH_3 the range in mg. of NH_3-N per 100 cc of medium in excess of control is shown. For convenience in discussing the results, each group of cultures having the same reactions have been given one letter.

It will be observed that groups A, B, C, and D comprised 79 of the 100 cultures, and of these 64 gave the beta type of hemolysis; in other words, these cultures were hemolytic on blood plates.

[5] Ibid., 1922, 30, p. 388.

TABLE 1

CHARACTERISTICS OF STREPTOCOCCI OF THE UDDER

Group	Number of Cultures	Morphology	Hemolysis	Methylene Blue Test	Litmus Milk Reaction		P_H in Fermentation							C c of CO_2 From		NH_3N Mg. per 100 C c Excess Over Control	Sodium Hippurate Hydrolyzed	
					37 C.	10 C.	Dextrose	Lactose	Saccharose	Salicin	Mannite	Raffinose	Inulin	Peptone	Dextrose			
A	38	Chains averaging 60-80 cells	Beta	—	Acid, coagulated, ⅛ decolorized	No change	4.5 +	4.5 +	4.5 +	7.3 —	7.3 —	7.3 —	7.3 —	Range 2.92-7.57 +	—	Range 20.32-24.38 +	+	Streptococcus mastitidis Var. Beta
B	26	Chains averaging 60-80 cells	Beta	—	Acid, coagulated, ⅛ decolorized	No change	4.5 +	4.5 +	4.5 +	4.5 +	7.3 —	7.3 —	7.3 —	3.25-5.91 +	—	22.14-23.54 +	+	
C	7	Chains averaging 60-80 cells	Gamma, some green	—	Acid, coagulated, ⅛ decolorized	No change	4.5 +	4.5 +	4.5 +	7.2 —	7.2 —	7.2 —	7.2 —	4.68-6.50 +	—	17.80-22.14 +	+	Var. Gamma
D	8	Chains averaging 60-80 cells	Gamma, some green	—	Acid, coagulated, ⅛ decolorized	No change	4.5 +	4.5 +	4.5 +	4.5 +	7.2 —	7.2 —	7.2 —	4.57-5.78 +	—	12.89-23.82 +	+	
E	3	Chains of 4-10 cells	Gamma, some green	+ 2-5 days	Acid	Slight acid	4.6 +	4.5 +	4.6 +	4.6 +	4.6 +	7.4 —	4.6 +	5.20-6.99 +	—	22.10-22.28 +	+	
F	2	Chains of 4-40 cells	Alpha weak	+	Acid	Slight acid	4.5 +	4.5 +	4.5 +	4.5 +	4.5 +	7.3 —	7.4 —	4.57-5.78 +	—	20.46-21.44 +	+	
G	4	Chains of 4-20 cells	Alpha weak	May decolorize after 5 days	Acid, may be slightly coagulated	No change	4.5 +	4.8 +	4.5 +	7.4 —	7.3 —	4.6 +	7.4 —	2.86-4.09 +*	—	0.0 - 1.69 —	±	
	4	Chains of 4-10 cells, sometimes longer	Alpha	—	No change	No change	6.2 +	6.2 +	6.2 +	7.3 —	7.3 —	7.5 —	7.5 —	2.60-3.18 +*	—	0.0-4.2 —	+	Streptococcus acidominimus and varieties
	6	Chains of 4-10 cells, sometimes longer	Alpha	—	No change	No change	6.2 +	6.2 +	6.2 +	6.0 +	7.3 —	7.5 —	7.5 —	3.25-10.27 +*	—	0.0-1.40 —	+	
	1	Long chains of hundreds of cells	Alpha	—	No change	No change	6.3 +	6.4 +	6.1 +	6.1 +	7.4 —	7.5 —	6.2 +	5.98 +*	—	0.56 —	+	
	1	Chains of 20-40 cells	Gamma	—	No change	No change	6.2 +	6.4 +	6.4 +	6.4 +	6.3 +	7.3 —	7.4 —	4.16 +*	—	0.0 —	+	

* Note that no NH_3 is formed.

The 79 cultures comprising the first 4 groups were all long-chain-forming streptococci. Groups A and B, 64 cultures, were hemolytic; while C and D were not, but were of the gamma or gamma-G type. The beta types varied in the fermentation of salicin, and the same may be said of the gamma types. The cultures of all 4 groups formed CO_2 and NH_3 from peptone, but no CO_2 from dextrose. Particular attention is called to the fact that they all hydrolyzed sodium hippurate, forming benzoic acid and glycocoll.

Because of our nonselective method of isolation, we feel that these streptococci represent the "majority streptococcus flora" of the cow's udder. They may be grouped and called Streptococcus mastitidis (Guillebeau). There are apparently two varieties, hemolytic and non-hemolytic, which may be termed beta and gamma varieties.

Nocard and Mollereau [6] in 1887 isolated a long-chain-forming streptococcus from a case of contagious mastitis; and from the rather meager description it would appear to be the common udder type encountered in our studies. It is interesting to observe that Nocard and Mollereau found that they could reproduce the disease by inoculation. The organism appears to have been termed Streptococcus mastitidis contagiosae by Guillebeau.[7] Jensen [8] has also applied the name streptococcus mastitidis to the organism causing mastitis.

STREPTOCOCCUS MASTITIDIS

Streptococcus mastitidis is characterized as follows: It does not reduce methylene blue; coagulates litmus milk usually in 24 hours, and partially decolorizes the milk after coagulation; does not grow in milk at 10 C. It ferments dextrose, lactose, and cane and may or may not ferment salicin. It does not ferment mannite, raffinose and inulin. It produces CO_2 and NH_3 from peptone, but no CO_2 from dextrose. It hydrolyzes sodium hippurate into benzoic acid and glycocoll. There are two varieties; one which produces the beta type of reaction, and the other which produces the gamma type on blood-agar plates.

Streptococcus mastitidis seems to agree in the ordinary cultural characteristics with the Streptococcus pyogenes type found to be common in the udder by Rogers and Dahlberg.[9] Sherman and Albus [10] also found the streptococcus pyogenes type, fermenting dextrose, lactose,

[6] Ann. Inst. Pasteur, 1887, 1, p. 109.
[7] Landw. Jahrb. Schweiz, 1890, 4, p. 27.
[8] Mem. d. l'Acad. Roy. d. sc. et Lettr. de Danemark, Sect. d. Sc., 1919, 5.
[9] U. S. Dept. Agr. Jour. Agr. Research, 1914, 1, p. 491.
[10] Jour. Bacteriol., 1918, 3, p. 153.

saccharose, and sometimes salicin, to be the characteristic streptococcus of the udder; and they pointed out the value of the negative reduction of methylene blue and the inability of this type to grow at 10 C. Jones [11] in a study of streptococci from the udder of cows having mastitis, found that they could be separated into 2 groups, one fermenting dextrose, lactose, maltose and saccharose, and the other salicin in addition. Both his hemolytic and nonhemolytic cultures fell in these 2 groups.

The "typical udder type," as we may term Streptococcus mastitidis, might be given various names according to various classifications. For example, by Holman's [12] scheme, group A could be called St. anginosus; group B, St. pyogenes; group C, St. salivarius; and group D, St. mitis.

Classification on this basis would, in our opinion, be incorrect, because it is based only on the fermentation of a few sugars and similar substances and hemolysis.

In view of the results obtained by the use of tests devised in these laboratories, such as the difference in P_H, the production of CO_2 and NH_3 from peptone, and the hydrolysis of sodium hippurate, we do not feel certain that the typical udder type is commonly distributed; nor are we sure that it is limited to the udder. This cannot be known until the differential tests are extensively applied to large numbers of streptococci from varied sources. We do believe that the typical udder type is not Streptococcus pyogenes.

According to Holman's classification, group B in table 1 would be called Streptococcus pyogenes, and so would the majority of human hemolytic types in our collection. In table 2 will be seen the principal cultural characteristics of 33 human hemolytic streptococci. Most of these were isolated from pathologic sources, with a few from normal throats.

On the basis of hemolysis and positive or negative fermentations the first 23 cultures would be identical with group B of the udder, but the use of other tests show that they are quite different.

Although both human and bovine types show the beta type of hemolysis and appear quite similar on blood plates, the human type is about 100 times more hemolytic than the bovine type, as shown by the hemolysis in tubes. This point has also been brought out by Brown.[13] In

[11] Jour. Exper. Med., 1918, 28, p. 149; ibid., p. 253.
[12] Jour. Med. Research, 1916, 34, p. 377.
[13] Jour. Exper. Med., 1920, 31, p. 35.

the methylene blue test and milk reaction there is little difference, although the human types are not quite so active in producing acidity in milk.

The P_H reached in the fermentation of test substances is distinctly different. The udder types reach about 4.5, while the human type produces less acid and reaches about P_H 5.5. This reaction was obtained in a dextrose yeast (Cerevisine) peptone broth. The final P_H can be varied, as has been shown by several investigators, but it is quite constant when the test is carried out in a definite medium.

Horry Jones [14] has shown that the hydrogen-ion concentration of human types of hemolytic streptococci can be increased; and F. S. Jones [15] has also shown that the so-called P_H limits can be changed by variation of the medium.

TABLE 2

CULTURAL CHARACTERISTICS OF HEMOLYTIC STREPTOCOCCI FROM HUMAN SOURCES, MOSTLY FROM PATHOLOGIC CONDITIONS

No. of Cultures	Hemolysis	P_H in Fermentations							CO_2 from		NH_3 from Peptone	Sodium Hippurate Hydrolyzed	Holman's Classification
		Dextrose	Lactose	Saccharose	Salicin	Mannite	Raffinose	Inulin	Peptone	Dextrose			
23	Beta	5.4 +	5.4 +	5.4 +	5.5 +	7.4 —	7.4 —	7.4 —	+	—	+	—	Streptococcus pyogenes
9	Beta	5.4 +	5.4 +	5.4 +	5.5 +	5.5 +	7.4 —	7.4 —	+	—	+	—	Streptococcus infrequens
1	Beta	4.6 +	4.6 +	4.6 +	4.6 +	7.2 —	4.5 +	7.1 —	+	—	+	—	

"The limiting P_H" has been the term commonly used, and it is defined by Jones [15] as the acid production in a given medium which limits the final growth. We believe that the term "final P_H" is less misleading than "the limiting P_H." The final P_H of a culture is not necessarily the limiting P_H, and we feel that the final P_H is that reached when growth ceases. It may be the limiting P_H in some cases, or it may be the result of double fermentations with a reversion of reaction as in the case of B. aerogenes. One is inclined to think of a limiting P_H, or the acid tolerance of an organism, as being quite a definite thing and not subject to much variation; while on the other hand, it is readily seen that the final P_H could be subject to variation due to the composition of the medium for growth.

[14] Jour. Infect. Dis., 1920, 26, p. 160.
[15] Jour. Exper. Med., 1920, 32, p. 273.

The final P_H of the streptococci and the difference between human and bovine types of hemolytic organisms have been discussed somewhat extensively, because we feel that this difference is quite fundamental and not of accidental occurrence.

Proceeding with the comparison of group B of the udder type and the 23 cultures of human type, it will be seen from tables 2 and 3 that both produce CO_2 and NH_3 from peptone and no CO_2 from dextrose. A further distinction between the two types is the negative hydrolysis of sodium hippurate by the human type and the positive reaction by the udder type. This test has been described by Ayers and Rupp.[5]

These differences in final P_H and in the hydrolysis of sodium hippurate hold for all udder types and human types, with the exception of one culture from a normal human throat. This culture reached a final P_H of about 4.6 but did not hydrolyze the hippurate. It also fermented raffinose. This type may be common in normal throats, but we have no information on this point, except that, as is generally known, raffinose fermenters are common in saliva.

CHARACTERISTICS OF STREPTOCOCCUS MASTIDITIS AND STREPTOCOCCUS PYOGENES

Streptococcus mastitidis, Var. Beta Var. Gamma		Streptococcus pyogenes
Beta Gamma type (weak) type	Blood-agar Plate	Beta type (strong)
* P_H about 4.5 in dextrose yeast broth	Dextrose	* P_H about 5.5 in dextrose-yeast broth
+	Lactose	+
+	Saccharose	+
(±)	Salicin	(±)
—	Mannite	—
—	Raffinose	—
—	Inulin	—
+	CO_2 from peptone	+
—	CO_2 from dextrose	—
+*	Sodium hippurate hydrolyzed	—*
Coagulated, partly decolorized after coagulation	Milk at 37 C.	Acid, may be coagulated
No growth	Milk at 10 C.	No growth
No decolorization	Methylene blue, milk	No decolorization

* Note particularly these reactions.

These differences between the streptococci of the udder and hemolytic streptococci from human sources have been discussed at length in order to show why we believe they are not the same species.

For the sake of clearness, we have listed the characteristics of what we consider Streptococcus mastitidis and also Streptococcus pyogenes.

Returning to table 1 again, it will be seen that groups E and F are not included in the Streptococcus mastitidis group. One group shows

the alpha type of hemolysis, and both reduce methylene blue, they form acid in milk, and grow at 10 C.; both ferment mannite, and one group ferments inulin. In the rest of their characteristics they agree with Streptococcus mastitidis.

Group G is distinctly different in that the organisms produce CO_2 in infusion peptone broth without NH_3. There were some indications that the CO_2 came from organic acid salts.

The 12 remaining cultures represent what appears to be a new species of streptococcus, which has been named merely as a matter of record. They generally showed the alpha type of hemolysis, which in most cases was very distinct. No change was observed in litmus milk, and they produced little acid in fermentation tests. This is the most characteristic feature of these organisms. In the yeast-peptone medium the P_H decreased from 7.5 to about 6.2. This medium was lightly buffered, and for this reason the weak fermentations were observed. There was considerable variation in the test substances fermented. These organisms also formed CO_2 from infusion-peptone broth; but no NH_3. Here again the CO_2 seemed to come from organic acid salts. Because of their ability to produce only a little acid from test substances, we have termed these cultures varieties of Streptococcus acidominimus. These organisms apparently are different from those observed by Holman [12] and called Streptococcus ignavius on account of their lack of fermentative ability.

STREPTOCOCCUS ACIDOMINIMUS

Streptococcus acidominimus is characterized by the small amount of acidity developed in the fermentation of test substances. The change in P_H is about 1.0- 1.5, say from 7.5 to 6.5 in a lightly buffered medium. Hemolysis is usually of the alpha type. No change is noted in the litmus milk. CO_2 is produced from a 4% peptone-infusion broth medium, but not from dextrose. No ammonia is formed as is usually the case when CO_2 is formed. The CO_2 probably comes from organic acid salts. Sodium hippurate is hydrolyzed to benzoic acid and glycocoll. There are several varieties of Streptococcus acidominimus which differ in the test substances fermented.

FREQUENCY OF THE PRESENCE OF STREPTOCOCCI IN THE UDDERS OF COWS

Streptococci occur frequently in the udders of normal cows. This has been shown by Sherman and Hastings,[16] Evans,[17] and also by

[16] Creamery and Milk Plant Monthly, 1915, 3, p. 11.
[17] Jour. Infect. Dis., 1916, 18, p. 437.

Jones.[18] In our studies streptococci have been isolated by direct platings of milk directly from the udder of 51 of the 133 normal cows examined. This represents about 38% of the animals tested. They were isolated from each of 17 cows having mastitis, and varied in 3 cases, from a few thousand to many millions. In some animals with mastitis the predominating streptococcus was the hemolytic (beta) variety and in other cases the nonhemolytic (gamma).

It seems evident that Streptococcus mastitidis is commonly found in the udders of normal cows, and also to a less extent other species of streptococcus. The fact that milk containing these organisms has been consumed regularly with no ill effects indicates that these streptococci need not be feared. In this connection the experiments of Nocard and Mollereau [6, 11] are of interest. They isolated a streptococcus, apparently Streptococcus mastitidis, and fed cultures to dogs and rabbits. No ill effects were noted, and they concluded that milk containing these organisms could be used for food without danger. Jones [11] also fed nonhemolytic udder streptococci to a pig, with no bad results.

SUMMARY AND CONCLUSIONS

1. The typical streptococcus of the udder of the cow was found to be Streptococcus mastitidis. Cultural characteristics of two varieties of this organism are described.

2. Streptococcus mastitidis is practically identical with Streptococcus pyogenes when the usual cultural characteristics are studied. They are separated largely on the difference in final P_H and difference in ability to hydrolyze sodium hippurate.

3. Cultural characteristics are presented of a few other streptococci which are not included with Streptococcus mastitidis.

4. An apparently new species is described which because of the small amount of acid produced in test substances is termed Streptococcus acidominimus.

5. It is shown that streptococci are frequently found in the udders of normal cows and that the same species are also present in cases of mastitis. There appears to be no reason to believe that Streptococcus mastitidis is pathogenic for man when consumed in milk, and it can apparently be readily distinguished from Streptococcus pyogenes.

[18] Jour. Exper. Med., 1918, 28, p. 735.